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Description and comparison of morphological structures of the eggs of *Anopheles* hyrcanus group and related species (Diptera: Culicidae) from the Republic of Korea

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Abstract

The eggs of six *Anopheles* Hyrcanus Group (*An. sinensis* Wiedemann, *An. kleini* Rueda, *An. belenrae* Rueda, *An. pullus* M. Yamada, *An. lesteri* Baisas and Hu, *An. sineroides* S. Yamada) and related species (*An. koreicus* S. Yamada and Watanabe, *An. lindesayi japonicus* S. Yamada), were described from scanning electron micrographs of specimens collected from different localities of the Republic of Korea. Morphometric measurements of egg samples of the eight species were compared and relationships analyzed by multivariate statistics. About 27 characters were selected and used as a basis for principal and discriminant function analyses. Scanning electron micrographs of various parts of the eggs were selected to illustrate interspecific differences for particular morphological features (e.g. anterior and posterior tubercles, decks, plastron, micropyles, floats).

Key words: Culicidae, *Anopheles*, mosquito eggs, morphology, Republic of Korea

Introduction

Anopheles Hyrcanus Group comprises several species which are vectors of malaria and other mosquito borne-diseases in the Oriental and Palearctic Regions. The group, as currently defined, includes about 42% of the species that comprised the Myzorhynchus Series of genus *Anopheles* Meigen subgenus *Anopheles* Meigen (Harbach 2004, Rueda 2005, Rueda *et al.* 2005). The group includes about 30 known species world-wide. Twenty eight of these species are found in the Oriental and Eastern Palearctic Regions and three in the Western Palearctic Region.

In the Republic of Korea (ROK), all eight known *Anopheles* species are under the subgenus *Anopheles* of genus *Anopheles*. Six of these species belong to the Hyrcanus Group, namely *An. belenrae* Rueda, *An. kleini* Rueda, *An. sinensis* Wiedemann, *An. sineroides* Yamada, *An. pullus* M. Yamada, and *An. lesteri* Baisas and Hu (Rueda 2005, Rueda *et al.* 2006). The other two species belong to the Barbirostris Group (*An. koreicus* Yamada and Watanabe) and the Lindesayi Group (*An. lindesayi japonicus* Yamada) (Tanaka *et al*. 1979). *Anopheles sinensis* is the most commonly collected anopheline throughout the ROK, followed by *An. kleini*, *An. pullus*, and *An. sineroides* (T.A. Klein, unpublished data). Preliminary data suggest that *An. pullus* and *An. kleini* are the primary vectors of *Plasmodium vivax* malaria near the demilitarized zone (DMZ) while *An.*

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14. ABSTRACT

The eggs of six Anopheles Hyrcanus Group (An. sinensis Wiedemann, An. kleini Rueda, An. belenrae Rueda, An. pullus M. Yamada, An. lesteri Baisas and Hu, An. sineroides S. Yamada) and related species (An. koreicus S. Yamada and Watanabe, An. lindesayi japonicus S. Yamada), were described from scanning electron micrographs of specimens collected from different localities of the Republic of Korea. Morphometric measurements of egg samples of the eight species were compared and relationships analyzed by multivariate statistics. About 27 characters were selected and used as a basis for principal and discriminant function analyses. Scanning electron micrographs of various parts of the eggs were selected to illustrate interspecific differences for particular morphological features (e.g. anterior and posterior tubercles, decks, plastron, micropyles, floats).

15. SUBJECT TERMS
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sinensis is a secondary vector (Lee *et al.* 2007). *Anopheles lesteri* (=*An. anthropophagus*) is a major vector of malaria in China; however, its vectorial capacity is unknown in the ROK. The other remaining four *Anopheles* species are not considered to be malaria vectors in the ROK.

Numerous studies (*e.g.* Linley *et al.* 1993, 1996, Junkum 2004) were conducted to compare the morphometry and morphology of various mosquito species from different locations. For members of the Hyrcanus Group, little is known about their egg morphology and morphometry, except the work of Linley *et al.* (1995) in Malaysia on *An. nigerrimus* Giles, *An. paraliae* Sandosham, *An. peditaeniatus* (Leicester), *An. sinensis* Wiedemann (probably *sensu lato*). Although the larvae, pupae and adults of the eight *Anopheles* species from the ROK are well described, nothing is known about their egg morphology.

The objective of this study was to provide preliminary descriptions and comparison of the egg morphological structures of Hyrcanus Group species and other *Anopheles* groups from the ROK.

Materials and methods

Specimen collection, rearing and identification. Collections of all species were conducted in Gyeonggi Province (including Inchon) and Chungcheongbuk Province, ROK in 2005, 2006 and 2008 (Figure 1). Larvae and pupae were collected from various habitats (e.g. rice paddies, ponds, stream margins, etc.). They were brought to the laboratory, and reared to adults. Emerged adults were identified using characters in Tanaka et al. (1979), and/or processed for PCR identification and sequencing. DNA was isolated from individual adult mosquitoes by phenol-chloroform extraction, and PCR or direct sequencing was carried out as described in Wilkerson *et al.* (2003) and Li *et al.* (2005). Adults obtained from field collected larvae or pupae were allowed to lay eggs in the laboratory, and were preserved in Bouin's solution after embryonation, approximately 36 hours. Additional adults that were bloodfed and resting in cow sheds, were also collected at selected locations in the ROK (Figure 1). These adults were placed in 500 ml screened cardboard cartons, provided with a 10% sucrose solution, and maintained in the laboratory at 26ºC $\pm$ 2ºC. Three days post-feeding, the females were anesthetized with ethyl acetate, a wing removed to induce oviposition, and then placed in a 200 ml plastic oviposition cup, ¼ filled with water. A sheet of paper was placed over the oviposition cup to retain the females. Following oviposition, the female was removed from the cup, placed in a cryovial, and stored at -70ºC until processed for PCR and sequences as described above.

Egg preservation and preparation for scanning electron microscopy (SEM). After oviposition, mosquito eggs were preserved in Bouin's solution ((Borror *et al.* 1989), with the following ingredients: ethyl alcohol (ETOH, 80%; 150 ml), formaldehyde (60 ml), glacial acetic acid (15 ml), and picric acid (1 gm.), for 5–10 days or until fixed. Eggs were then washed 6–8 times in 70% ETOH, transferred to glutaraldehyde (GTA, 4%), and again washed 6–8 times in 70% ETOH, followed by dehydration in ETOH: 70%, 80%, 95%, and 100% for 10 minutes each. For critical point drying, the egg samples were sandwiched between two layers of 10µm polycarbonate membrane with a spacer in between and then transferred to two changes of 100% ETOH from 15 minutes to 2 hours. Eggs were loaded into the critical point drier (CPD), then kept at 10ºC with fresh carbon dioxide (CO_2) exchanged at least four times on 15-minute intervals to replace the ETOH before heating to 40ºC, just above the critical point temperature, and slowly depressurized. Another modified technique (see below) using the same sandwich method was also used to find a better critical point drying, but no apparent differences in results between the two methods were observed. Some batches of egg samples were transferred to two changes of 100% ETOH from 15 minutes to 2 hours. Samples were then transferred to 100% purified amyl acetate (CAS no. 628-63-7; Mallinckrodt Baker, Philipsburg, NJ) for two changes from 15 minutes to 2 hours. Samples were loaded into the CPD, kept at 10ºC with fresh CO_2 exchanged at least four times on 15-minute intervals to replace the amyl acetate before heating to 40ºC, just above the critical point temperature, and then slowly depressurized. After the critical point drying process, the eggs were positioned on stubs covered with sticky tape, and sputter-coated with gold. They were examined

and microphotographed using Philips XL-30 SESEM (FEI Co., Hillsboro, Oregon), at the Smithsonian Institution SEM laboratory, Washington, D.C.

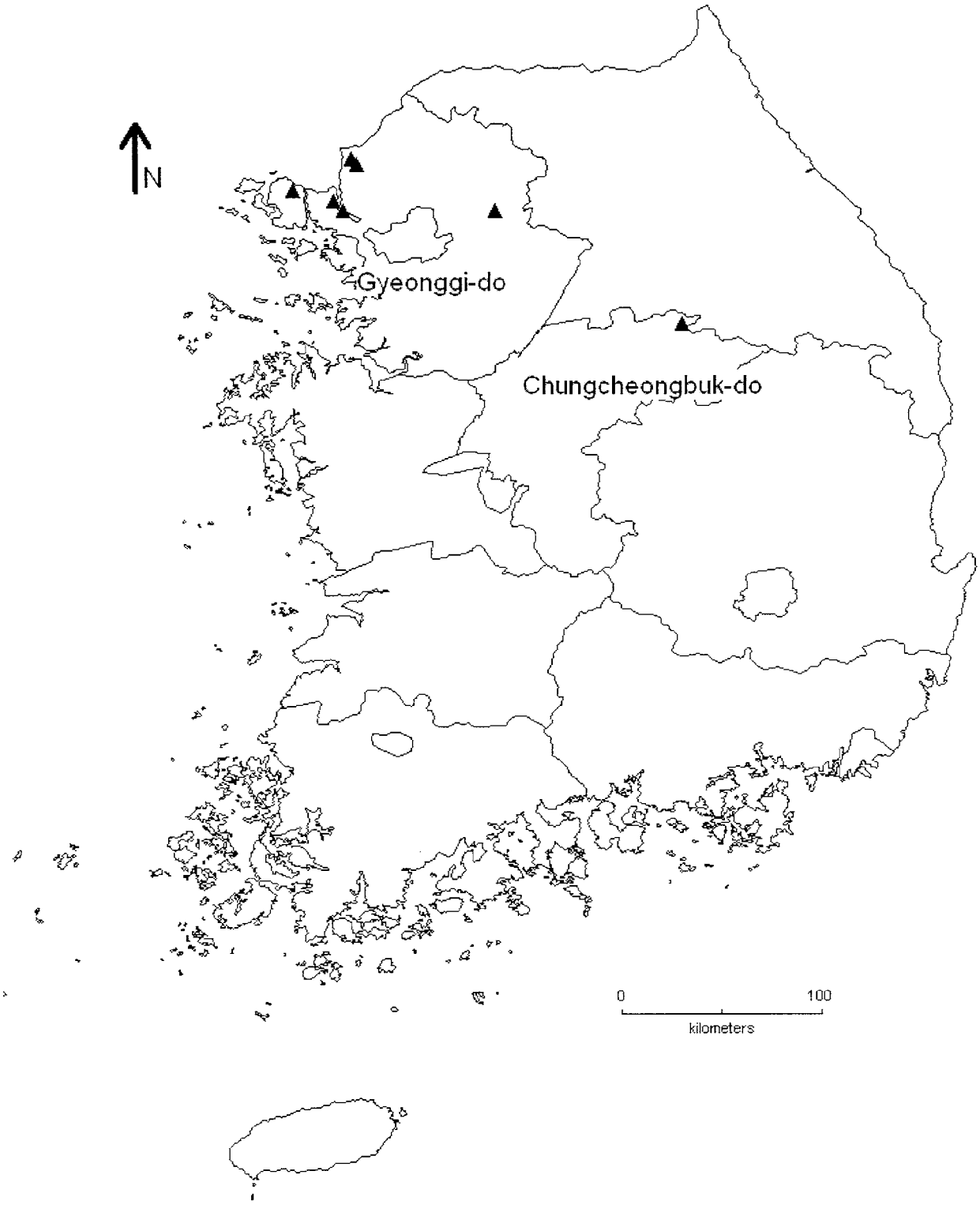

FIGURE 1. Map of the Republic of Korea showing collection sites (triangles) of *Anopheles* species from Gyeonggi Province (including Inchon Metropolitan Area) and Chungcheongbuk Province.

Data acquisition and analysis. The terminology of Linley *et al.* (1996) and Harbach and Knight (1980; except their float ridges for ribs) is used for the morphological characters or attributes. Definitions of abbreviations (acronyms) of measured or calculated attributes of *Anopheles* eggs are shown in the Appendix.

For each species, measurements were made from microphotographs compiled from 10–30 eggs. Twenty-seven attributes (Table 1) were recorded or derived (percentages or ratios) from the ventral and/or dorsal surfaces of the whole egg. The general descriptive terminology follows Harbach and Knight (1980) or Linley *et al.* (1993). The list of acronyms used in this work is shown in the Appendix. Variations in attributes among the species was examined by analyses of variance performed by the ANOVA procedure of SAS (SAS Institute 2003), and significant differences among means detected by the Ryan-Einot-Gabriel-Welsh multiple-range test (REGWQ). Twenty seven attributes were selected for multivariate analyses to detect levels of differentiation among species. Discriminant function analysis and principal components analysis were performed with the discriminant procedures and PRINCOMP, respectively (Statpoint Technologies 2007, SAS 2003) using default settings for the latter.

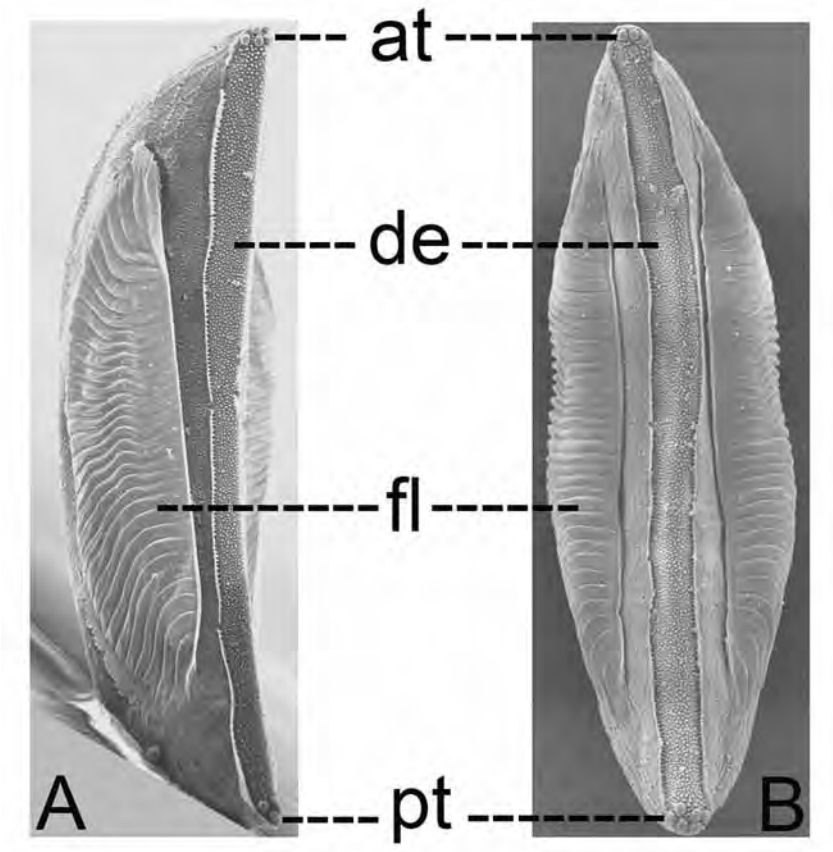

FIGURE 2. Lateral (A) and ventral (B) views of *An. sineroides* egg, showing general morphological parts, 200x. Abbreviations used include **at** = anterior tubercles, **de** = deck, **fl** = float (with ribs), **pt** = posterior tubercles.

Results and discussion

Descriptions of eggs of eight *Anopheles (Anopheles)* species from the Republic of Korea.

1. *Anopheles kleini* Rueda

(Figs. 3A, 4A, 5A, 6A)

Size: Length 349.66–525.23 um (mean 476.34 ± 73.38 um, n = 5); width 98.12–121.36 (mean 115.81 ± 9.96 um, n = 5) (Table 1). *Color:* Black. *Overall appearance*: Boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end blunt, sometimes pointed. Ventral surface concave, dorsal surface curved, float relatively short and wide in dorso-ventral plane, length 189.53–310.07 um (mean 243.44 ± 47.23 um, n =

5); width 41.44–60.80 um (mean 53.90 ± 8.74 um, n = 5). *Dorsal and lateral surfaces*: All surfaces uniformly covered with mostly pentagonal and hexagonal outer chorionic cells or plastron-type cells (Hinton 1968) (Fig. 3A), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with fine rounded structures, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 206.78–360.12 um (mean 293.32 ± 49.92, n = 16) (Table 1). Float fairly short, about 0.51 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs (= ridges, Harbach and Knight 1980) towards both ends of float wider than those at middle part, rarely striated on dorsal sides; number of ribs per float 20–25 (mean 23.20 ± 1.30, n = 5). *Ventral surface.* Deck continuous, narrows in mid-line near center of float, degree of narrowing usually variable; anterior part of deck usually wider than posterior part; entire deck covered uniformly with fine tubercles (Fig. 4A). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 6–7 (mean 3.67 ± 0.58, n = 3), and at posterior end, 8 (n = 3) (Table 1, Fig. 5A). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 6–9 (mean 7.31 ± 0.85, n = 13); lobes of each posterior ventral tubercle, 3 (n = 3). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, tuberculoid structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6A). Number of sectors (or ridges) 6–8 (mean 7.25 ±96, n = 4). Area of micropylar disc 33.98–146.76 um (mean 89.53 ± 51.45 um, n = 4), usually with striated surface.

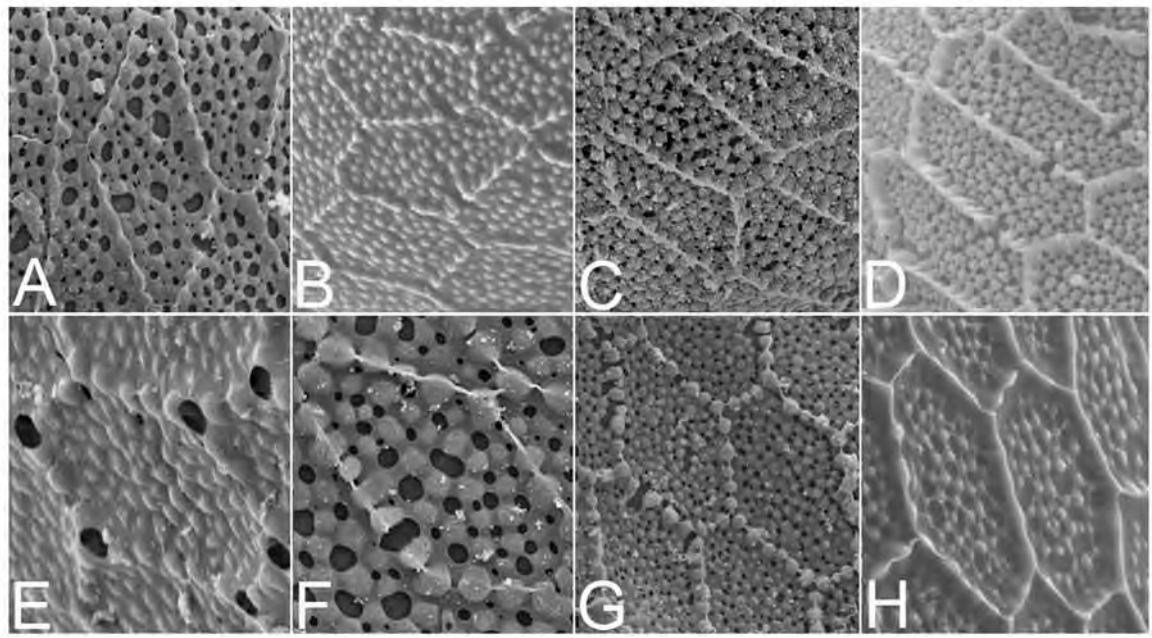

FIGURE 3. Anterior dorsal plastron of *Anopheles (Anopheles)* species. A, *An. kleini;* B, *An. sinensis*; C, *An. sineroides*; D, *An. pullus*; E, *An. lindesayi japonicus;* F, *An. koreicus;* G, *An. lesteri;* H, *An. belenrae.* Magnification, 3500x.

2. *Anopheles sinensis* Wiedemann
(Fig. 3B, 4B, 5B, 6B)

Size: Length 315.63–536.50 um (mean 467.37 ± 68.90 um, n = 10); width 52.43–145.00 (mean 76.72 ± 30.7 um, n = 8) (Table 1). *Color:* Black. *Overall appearance*: Boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end blunt, sometimes pointed. Ventral surface concave, dorsal surface curved, float relatively short and wide in dorso-ventral plane, length 147.42–298.20 um (mean 246.98 ± 49.22 um, n = 7); width 47.25–96.25 um (mean 67.08 ± 16.25 um, n = 7). *Dorsal and lateral surfaces*: All surfaces

uniformly covered with mostly hexagonal and pentagonal, but occasionally quadrilateral outer chorionic cells or plastron-type cells (Fig. 3B), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 131.87–273.40 um (mean 196.03 ± 50.42, n = 26) (Table 1). Float fairly short, about 0.53 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, rarely striated on dorsal sides; number of ribs per float 20–26 (mean 23 ± 1.63, n = 13). *Ventral surface.* Deck continuous, narrows in mid-line near center of float, degree of narrowing usually variable; covered uniformly with fine tubercles (Fig. 4B). Middle part of deck sometimes covered with thin membranous layer. Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the decks, 5–8 (mean 6.73 ± 1.16, n = 15), and at posterior end, 4–8 (mean 6.43 ± 1.51, n = 7) (Table 1, Fig. 5B). Lobed ventral tubercles usually oval or oblong, but occasionally round. Lobes of each anterior ventral tubercle, 5–8 (mean 6.92 ± 1.0, n = 24); lobes of each posterior ventral tubercle, 4–9 (mean 7.22 ± 1.1, n = 41). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, striated structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about a third or half way across micropylar disc, dividing disc in to sectors (Fig. 6B). Number of sectors (or ridges) 6–9 (mean 7.3 ± 1.0, n = 9). Area of micropylar disc 90.25–111.45 um (mean 100.69 ± 13.16 um, n = 3), usually with smooth surface.

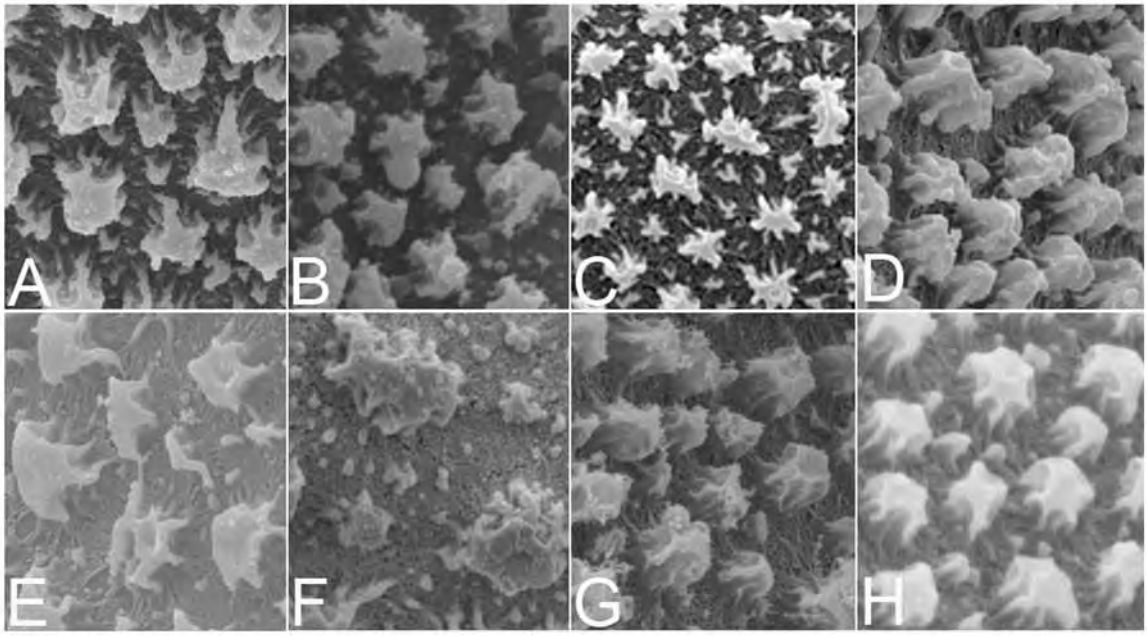

FIGURE 4. Chronic tubercles of middle part of deck of *Anopheles (Anopheles)* species. A, *An. kleini;* B, *An. sinensis*; C, *An. sineroides*; D, *An. pullus*; E, *An. lindesayi japonicus;* F, *An. koreicus;* G, *An. lesteri;* H, *An. belenrae.* Magnification, 3500x.

3. *Anopheles sineroides* S. Yamada
(Fig. 2A, B, 3C, 4C, 5C, 6C)

Size: Length 528.25–584.94 um (mean 553.93 ± 24.22 um, n = 7); width 70.12–140.16 (mean 96.71 ± 27.16 um, n = 5) (Table 1, Fig. 2A, B). *Color:* Black. *Overall appearance*: Slightly boat-shaped in both ventral and dorsal views, anterior and posterior ends blunt, sometimes slightly pointed. Ventral surface slightly concave, dorsal surface curved, float relatively long and wide in dorso-ventral plane, length 367.40–424.75 um (mean 401.20 ± 22.83 um, n = 5); width 59.20–100.10 um (mean 73.97 ± 16.21 um, n = 5). *Dorsal and lateral*

surfaces: All surfaces uniformly covered with mostly pentagonal and hexagonal outer chorionic cells or plastron-type cells. (Fig. 3C), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 78.84–330.18 um (mean 202.88 ± 69.07, n = 20) (Table 1). Float fairly long, about 0.72 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, slightly striated on dorsal sides; number of ribs per float 28–36 (mean 28.56 ± 3.00, n = 9). *Ventral surface.* Deck continuous, slightly narrows at both ends of float, degree of narrowing usually variable; anterior part of deck usually as wide as posterior part; entire deck covered uniformly with fine tubercles (Fig. 4C). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 5–6 (mean 5.50 ± 0.71, n = 2), and at posterior end, 3–4 (mean 3.60 ± 0.55, n = 5), (Table 1, Fig. 5C). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 5–7 (mean 6.50 ± 0.84, n = 6); lobes of each posterior ventral tubercle, 6–7 (mean 6.33 ± 0.58, n = 3). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, tuberculoid structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6C). Number of sectors (or ridges) 6–7 (mean 6.60 ± 0.55, n = 5). Area of micropylar 60.35–90.67 um (mean 80.01 ± 17.04 um, n = 3), usually with smooth surface, or covered with thin film.

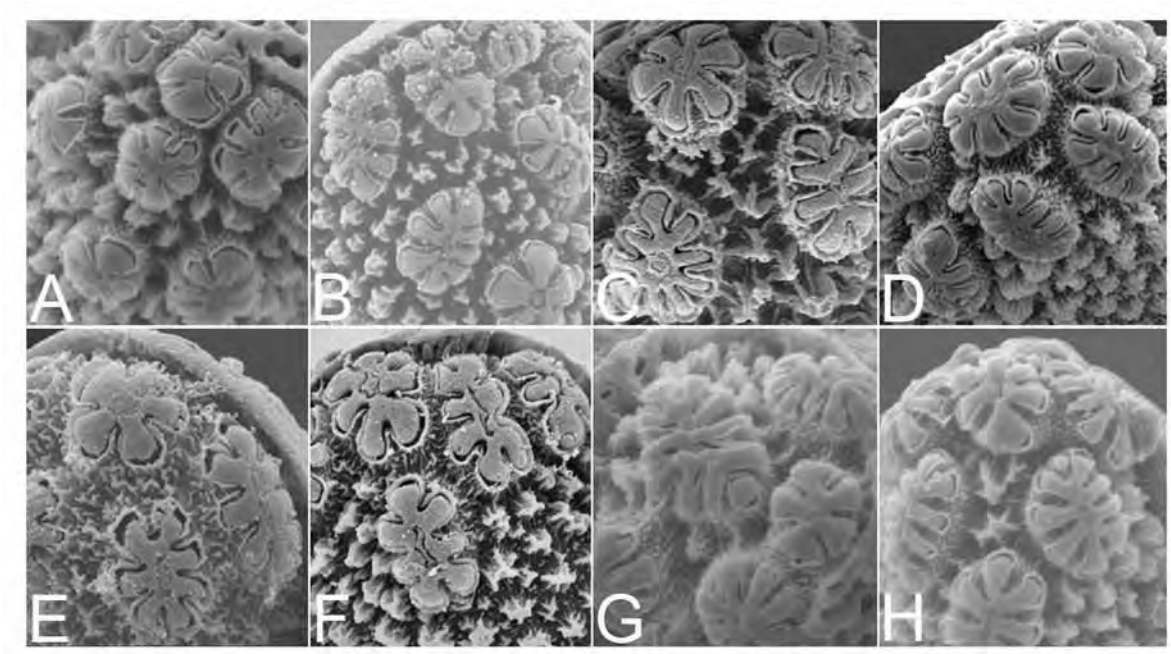

FIGURE 5. Anterior ventral tubercles of *Anopheles (Anopheles)* species. A, *An. kleini;* B, *An. sinensis*; C, *An. sineroides*; D, *An. pullus*; E, *An. lindesayi japonicus;* F, *An. koreicus;* G, *An. lesteri;* H, *An. belenrae.* Magnification, 3500x.

4. *Anopheles pullus* M. Yamada
(Fig. 3D, 4D, 5D, 6D)

Size: Length 447.76–551.20 um (mean 497.04 ± 28.83 um, n = 15); width 64.26–124.80 (mean 97.96 ± 21.66 um, n = 12) (Table 1). *Color:* Black. *Overall appearance*: Slightly boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end slightly pointed, sometimes blunt. Ventral surface slightly concave, dorsal surface curved, float relatively short and wide in dorso-ventral plane, length 244.95–499.72 um (mean

318.18 ± 80.20, n = 14); width 35.70–87.36 um (mean 58.83 ± 14.10 um, n = 14). *Dorsal and lateral surfaces*: All surfaces uniformly covered with quadrilateral, hexagonal, and mostly pentagonal outer chorionic cells or plastron-type cells. (Fig. 3D), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 100.80–340.80 um (mean 236.64 ± 64.82, n = 21) (Table 1). Float fairly short, about 0.57 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, slightly striated on dorsal sides; number of ribs per float 19–27 (mean 24.38 ± 2.03, n = 16). *Ventral surface.* Deck continuous, slightly narrows at middle of float, degree of narrowing usually variable; anterior part of deck usually as wide as posterior part; entire deck covered uniformly with fine tubercles (Fig. 4D). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 4–8 (mean 6.36 ± 1.36, n = 11), and at posterior end, 4–8 (mean 5.40 ± 1.17, n = 10), (Table 1, Fig. 5D). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 5–11 (mean 7.74 ± 1.20, n = 43); lobes of each posterior ventral tubercle, 3–11 (mean 7.65 ± 1.52, n = 31). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, tuberculoid structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6D). Number of sectors (or ridges) 7–8 (mean 7.50 ± 0.71, n = 2). Area of micropylar disc 66.61–109.59 um (mean 88.10 ± 30.39 um, n = 2), usually with striated surface.

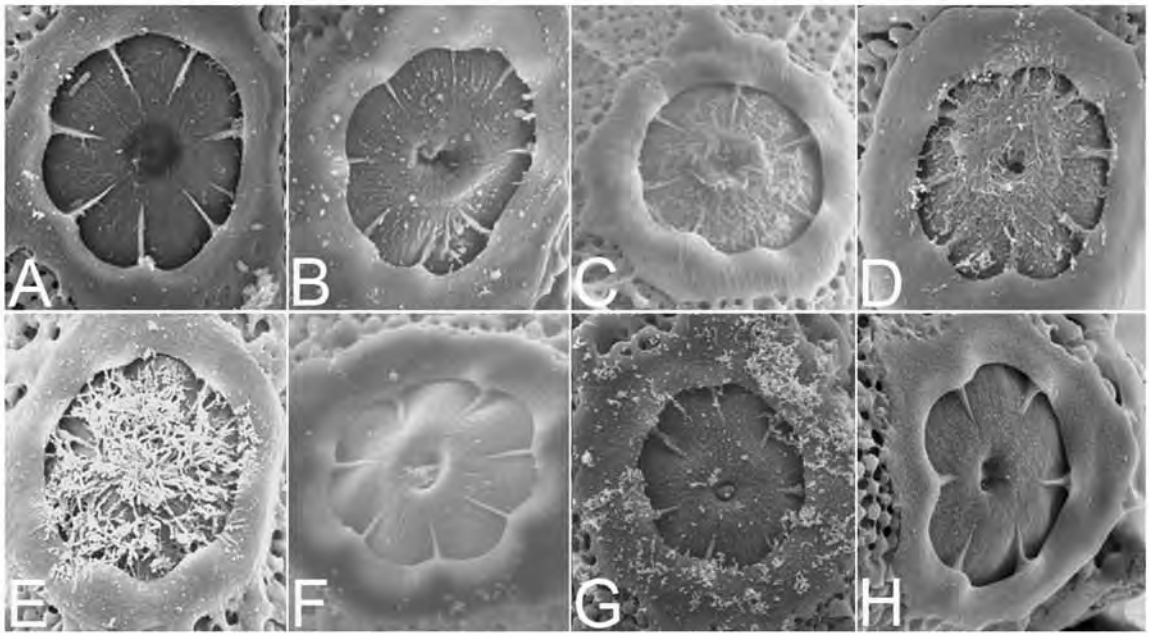

FIGURE 6. Dorsal micropylar discs of *Anopheles (Anopheles)* species. A, *An. kleini;* B, *An. sinensis*; C, *An. sineroides*; D, *An. pullus*; E, *An. lindesayi japonicus*,F, *An. koreicus,* G, *An. lesteri,* H, *An. belenrae.* Magnification, 3500x.

5. *Anopheles lindesayi japonicus* S. Yamada
(Fig. 3E, 4E, 5E, 6E)

Size: Length 495.72–546.28 um (mean 522.15 um, n = 5); width 62.10–93.43 (mean 73.82 ± 12.21 um, n = 5) (Table 1). *Color:* Black. *Overall appearance*: Slightly boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end slightly pointed, sometimes blunt. Ventral surface slightly concave, dorsal surface curved, float relatively short and wide in dorso-ventral plane, length 62.10–93.43 um (mean 73.82 ± 12.21

um, n = 5); width 88.14–110.70 um (mean 102.72 ± 10.23 um, n = 5). *Dorsal and lateral surfaces*: All surfaces uniformly covered with mostly pentagonal and hexagonal outer chorionic cells or plastron-type cells. (Fig. 3E), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 114.15–116.13 um (mean 115.13 ± 99.11, n = 3) (Table 1). Float fairly long, about 0.76 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float slightly wider than those at middle part, slightly striated on dorsal sides; number of ribs per float 24–28 (mean 25.00 ± 1.55, n = 6). *Ventral surface*. Deck continuous, slightly narrows at both ends of float, degree of narrowing usually variable; anterior part of deck usually slightly wider than posterior part; entire deck covered uniformly with fine tubercles (Fig. 4E). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 5 (n = 2), and at posterior end, 4 (n = 4), (Table 1, Fig. 5E). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 5–9 (mean 7.07 ± 1.07, n = 14); lobes of each posterior ventral tubercle, 4–7 (mean 5.20 ± 1.10, n = 5). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, striated structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6E). Number of sectors (or ridges) 7 (n = 3). Area of micropylar disc 114.15–116.13 um (mean 115.13 ± 99.11 um, n = 3), usually with striated surface.

6. *Anopheles koreicus* Yamaha & Watanabe
(Fig. 3F, 4F, 5F, 6F)

Size: Length 423.86–494.24 um (mean 454.16 ± 34.16 um, n = 4); width 117.19–133.03 (mean 125.06 ± 7.92 um, n = 3) (Table 1). *Color:* Black. *Overall appearance*: Boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end slightly pointed, sometimes blunt. Ventral surface slightly concave, dorsal surface strongly curved, float relatively short and wide in dorso-ventral plane, length 258.90–375.28 um (mean 332.89 ± 51.97 um, n = 4); width 67.36–130.10 um (mean 87.81 ± 28.68 um, n = 4). *Dorsal and lateral surfaces*: All surfaces uniformly covered with pentagonal and mostly hexagonal outer chorionic cells or plastron-type cells. (Fig. 3F), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 119.51–380.18 um (mean 249.09 ± 102.75, n = 17) (Table 1). Float fairly long, about 0.73 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, slightly striated on dorsal sides; number of ribs per float 26–35 (mean 30.40 ± 4.16, n = 5). *Ventral surface*. Deck continuous, slightly widens at both ends of float, degree of widening usually variable; anterior end of deck usually wider than posterior end. Entire deck usually covered by thin ornamented layer, having elongate openings at both ends extending towards middle, with or without round opening at middle; very fine tubercles on deck fairly visible through those layer openings ((Fig. 4F). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 3–4 (mean 3.67 ± 0.58, n = 3), and at posterior end, 3–4 (mean 3.25 ± 0.50, n = 4), (Table 1, Fig. 5F). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 6–9 (mean 8.00 ± 1.00, n = 16); lobes of each posterior ventral tubercle, 5–9 (mean 6.67 ± 1.15, n = 12). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, striated structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6F). Number of sectors (or ridges) 7 (n = 4). Area of micropylar disc 94.11–111.01 um (mean 103.02 ± 8.51 um, n = 5), usually with smooth or finely striated surface.

TABLE 1. Attributes of eggs of eight species of *Anopheles (Anopheles)* collected from various sites in the Republic of Korea.*

	ANOPHELES SPECIES														
	BELENRAE				KLEINI			KOREICUS				LESTERI			
Attribute	n	Mean	SD		n	Mean	SD		n	Mean	SD		n	Mean	SD	
Linear dimensions																
EGGL	5	572.07	40.44	a	5	476.34	73.38	c	4	454.16	34.16	c	7	486.96	33.91	bc
EGGW	5	129.09	40.65	a	5	115.81	9.96	ab	3	125.06	7.92	a	7	75.68	9.40	bc
ELWR	5	5.06	2.51	abc	5	4.15	0.81	bc	3	3.70	0.80	c	7	6.51	0.81	ab
Float attributes																
FLOL	5	293.80	15.59	bc	5	243.44	47.23	c	4	332.89	51.97	abc	8	355.49	30.44	ab
FLOW	5	74.44	22.25	abc	5	53.90	8.74	c	4	87.81	28.68	ab	8	80.47	16.80	abc
FRIN	8	23.63	2.20	b	5	23.20	1.30	b	5	30.40	4.16	a	8	31.50	2.62	a
FLOA	5	21886.56	7139.03	bc	4	13128.75	3657.05	c	4	29456.69	10887.24	b	8	28379.26	4710.02	b
FLWR	5	4.14	0.91	ab	4	4.82	1.17	ab	4	4.00	1.05	ab	8	4.58	0.96	ab
FLELP	5	51.37	5.52	a	5	51.48	8.50	a	4	73.08	8.07	a	7	73.86	4.08	a
FLEWR	5	2.65	1.52	cd	5	2.14	0.62	d	3	2.64	0.38	cd	7	4.81	0.66	ab
FLRR	5	12.31	1.63	bc	5	10.57	2.36	c	4	11.30	1.97	bc	8	11.38	1.57	bc
FWEWR	5	0.64	0.29	bc	5	0.47	0.07	c	3	0.59	0.01	bc	7	1.07	0.38	ab
FWRR	5	3.13	0.95	ab	5	2.34	0.47	b	4	2.91	0.55	ab	8	2.55	0.40	b
Deck attributes																
DECL	2	527.48	12.33	ab	2	427.11	110.44	b	3	462.00	36.10	ab	5	518.67	56.95	ab
DECW	2	59.42	1.05	bc	2	77.80	13.37	a	3	43.24	5.04	dc	5	35.51	8.04	d
DEMW	3	34.49	5.35	b	2	29.43	0.67	b	3	37.05	3.69	b	5	49.33	15.47	ab
DECA	4	20320.42	7431.08	bc	2	32490.16	2879.93	ab	3	19974.07	2865.67	bc	4	18837.25	5420.40	c
Ventral tubercles																
ATUN	4	7.75	2.50	ab	3	6.67	0.58	abc	3	3.67	0.58	c	3	8.33	1.53	a
PTUN	3	5.00	1.73	bc	3	8.00	0.00	a	4	3.25	0.50	c	4	5.50	2.38	bc
ATLN	22	7.50	1.41	a	13	7.31	0.85	a	16	8.00	1.00	a	19	7.21	1.58	a
PTLN	3	3.33	0.58	b	3	3.00	0.00	b	12	6.67	1.15	a	24	7.50	1.84	a
Micropyle and plastron																
MICA	3	20.98	0.50	b	4	89.53	51.45	a	5	103.02	8.51	a	3	103.79	2.17	a
DPDN	14	5.57	0.51	ab	16	5.56	0.51	ab	17	5.59	0.51	ab	20	5.60	0.50	ab
DPLA	14	264.84	66.08	ab	16	293.32	49.92	a	17	249.09	102.75	abc	20	261.30	67.04	ab
DPNLR	14	48.28	13.79	ab	16	53.30	11.08	a	17	44.51	17.59	ab	20	46.98	12.64	ab
MICDR	3	2.66	0.01	b	4	12.20	6.99	b	5	14.71	1.21	b	3	14.89	0.20	b
MIDN	4	7.25	0.96	a	4	7.25	0.96	a	5	7.00	0.00	a	3	6.00	1.00	a

*For definition of abbreviations, see Appendix. Unit of measurements: length and width, um; area, sq. um.

Means in the same row followed by the same letter are not significantly different ($P \leq 0.05$, Ryan-Einot-Gabriel-Welsh multiple-range test , REGWQ).

continued next page

TABLE 1. (continued)

	LINDESAYI				PULLUS				SINENSIS				SINEROIDES		
Attribute	n	Mean	SD		n	Mean	SD		n	Mean	SD		n	Mean	SD	
Linear dimensions																
EGGL	5	522.15	22.07	abc	15	497.04	28.83	bc	10	487.37	44.76	bc	5	553.93	24.22	ab
EGGW	5	73.82	12.21	b	12	97.76	21.66	ab	8	76.72	30.71	b	5	96.71	27.16	ab
ELWR	5	7.19	0.94	a	12	5.39	1.18	abc	8	6.47	1.65	ab	5	6.02	1.32	abc
Float attributes																
FLOL	5	73.82	12.21	a	14	318.18	80.20	abc	7	246.98	49.32	c	5	401.20	22.83	a
FLOW	5	102.72	10.23	a	14	58.83	14.10	bc	7	67.08	16.25	bc	5	73.97	16.21	abc
FRIN	6	25.00	1.55	b	16	24.38	2.03	b	13	23.00	1.63	b	9	28.56	3.00	a
FLOA	5	40616.64	3470.53	a	12	16600.07	5492.05	c	7	16922.83	5861.57	c	5	29430.80	4869.71	b
FLWR	5	3.89	0.47	b	12	4.99	0.87	ab	7	3.76	0.71	b	5	5.65	1.29	a
FLELP	5	75.89	2.18	a	11	57.40	3.67	a	7	53.42	6.30	a	5	72.42	2.43	a
FLEWR	5	5.46	0.76	a	10	3.61	1.09	bcd	6	3.54	1.15	bcd	5	4.36	0.95	bcd
FLRR	4	15.50	0.80	a	12	12.11	1.31	bc	7	11.18	2.44	bc	5	14.12	1.76	ab
FWEWR	5	1.44	0.33	a	10	0.65	0.13	bc	6	0.88	0.23	bc	5	0.84	0.38	bc
FWRR	4	4.13	0.66	a	14	2.44	0.63	b	7	2.99	0.92	ab	5	2.65	0.86	b
Deck attributes																
DECL	5	515.08	34.34	ab	7	501.73	46.71	ab	3	323.57	211.23	ab	4	558.22	22.80	ab
DECW	5	27.62	6.61	d	7	42.61	10.20	dc	3	73.66	6.97	ab	4	39.87	4.36	d
DEMW	5	55.52	14.34	ab	3	50.10	11.65	ab	4	67.54	12.04	a	4	36.55	3.41	b
DECA	5	14147.42	3023.84	c	5	22337.58	6958.14	abc	3	34709.21	6147.77	a	3	23293.48	1679.67	abc
Ventral tubercles																
ATUN	2	5.00	0.00	c	11	6.36	1.36	abc	15	6.73	1.16	abc	2	5.50	0.71	abc
PTUN	4	4.00	0.00	bc	10	5.40	1.17	bc	7	6.43	1.51	ab	5	3.60	0.55	ab
ATLN	14	7.07	1.07	a	43	7.74	1.20	a	24	6.92	0.97	a	6	6.50	0.84	a
PTLN	5	5.20	1.10	ab	31	7.65	1.52	a	41	7.22	1.13	a	3	6.33	0.58	a
Micropyle and plastron																
MICA	3	115.13	99.11	a	2	88.10	30.39	a	3	100.69	13.16	a	3	80.01	17.04	a
DPDN	14	5.90	0.31	a	21	5.20	0.93	b	26	5.42	0.70	ab	23	5.57	0.59	ab
DPLA	14	300.73	57.93	a	21	236.64	64.82	abc	26	196.03	50.42	c	23	202.88	69.07	bc
DPNLR	14	51.06	9.94	a	21	46.18	11.03	ab	26	36.80	10.77	b	23	36.48	11.88	b
MICDR	3	16.45	7.92	a	2	11.60	2.95	b	3	14.38	1.88	b	3	12.85	3.66	b
MIDN	4	7.00	0.00	a	2	7.50	0.71	a	9	7.33	1.00	a	5	6.60	0.55	a

*For definition of abbreviations, see Appendix. Unit of measurements: length and width, um; area, sq. um.

Means in the same row followed by the same letter are not significantly

7. *Anopheles lesteri* Baisas & Hu
(Fig. 3G, 4G, 5G, 6G)

Size: Length 423.57–537.25 um (mean 486.96 ± 33.91 um, n = 7); width 60.04–85.68 (mean 75.68 ± 9.40 um, n = 7) (Table 1). *Color:* Black. *Overall appearance*: Slightly boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end slightly pointed, sometimes blunt. Ventral surface slightly concave, dorsal surface curved, float relatively long and wide in dorso-ventral plane, length 318.25–402.50 um (mean 355.49 ± 30.44 um, n = 8); width 65.45–115.14 um (mean 80.47 ± 16.80 um, n = 8). *Dorsal and lateral surfaces*: All surfaces uniformly covered with mostly pentagonal and hexagonal outer chorionic cells or plastron-type cells. (Fig. 3G), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with perforated meshwork, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 163.19–380.20 um (mean 261.30 ± 67.04, n = 20) (Table 1). Float fairly long, about 0.74 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, slightly striated on dorsal sides; number of ribs per float 28–37 (mean 31.50 ± 2.62, n = 8). *Ventral surface.* Deck continuous, slightly narrows at both ends of float, degree of narrowing usually variable; anterior part of deck usually as wide as posterior part; entire deck covered uniformly with fine tubercles (Fig. 4G). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 7–10 (mean 8.33 ± 1.53, n = 3), and at posterior end, (mean 5.50 ± 2.38, n = 4), (Table 1, Fig. 5G). Lobed ventral tubercles usually round, occasionally oval or oblong. Lobes of each anterior ventral tubercle, 4–10 (mean 7.21 ± 1.58, n = 19); lobes of each posterior ventral tubercle, 4–11 (mean 7.50 ± 1.84, n = 24). Lobes clearly separated, often swollen at ends, outer walls often smooth. Lobes in slightly elevated, striated structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with smooth surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6G). Number of sectors (or ridges) 6–8 (mean 7.25 ± 0.96, n = 4). Area of micropylar disc 33.98–146.76 um (mean 89.53 ± 51.45 um, n = 4), usually with striated surface.

8. *Anopheles belenrae* Rueda
(Figs. 3H, 4H, 5H, 6H)

Size: Length 518.76–604.87 um (mean 572.07 ± 40.44 um, n = 5); width 66.51–150.62 (mean 129.09 ± 40.65 um, n = 5) (Table 1). *Color:* Black. *Overall appearance*: Boat-shaped in both ventral and dorsal views, anterior end blunt, posterior end blunt, sometimes pointed. Ventral surface concave, dorsal surface curved, float relatively short and wide in dorso-ventral plane, length 266.87–304.33 um (mean 293.80 ± 15.59 um, n = 5); width 57.86–111.08 um (mean 74.44 ± 22.25 um, n = 5). *Dorsal and lateral surfaces*: All surfaces uniformly covered with mostly pentagonal and hexagonal outer chorionic cells or plastron-type cells (Hinton 1968) (Fig. 3H), each longer than wide, long dimension oriented in long axis of egg. Interior of each cell with interconnected, fine rounded structures, surrounded by an elevated, palisade-like outer chorionic reticulum. Cell area 98.38–374.86 um (mean 264.84 ± 66.08, n = 14) (Table 1). Float fairly short, about 0.51 length of egg; ratio of float length and width, and length in proportion to egg length and number of ribs as in Table 1. Ribs towards both ends of float wider than those at middle part, rarely striated on dorsal sides; number of ribs per float 21–28 (mean 23.63 ± 2.20, n = 8). *Ventral surface.* Deck continuous, narrows in mid-line near center of float, degree of narrowing usually variable; anterior part of deck usually wider than posterior part; entire deck covered uniformly with fine tubercles (Fig. 4H). Frill continuous, shallow along narrowed portion of deck. Lobed ventral tubercles at anterior end of the deck, 4–9 (mean 7.75 ± 2.50, n = 4), and at posterior end, 4–7 (mean 5.00 ± 1.73, n = 3) (Table 1, Fig. 5H). Lobed ventral tubercles usually oval or oblong, occasionally round. Lobes of each anterior ventral tubercle, 4–10 (mean 7.50 ± 1.41, n = 22; lobes of each posterior ventral tubercle, 3–4 (mean 3.33 ± 0.58, n = 3). Lobes clearly separated, often swollen at ends, outer walls often

smooth. Lobes in slightly elevated, tuberculoid structures. *Anterior end, micropyle*. Anterior end slightly more blunt than posterior end. Micropylar collar irregular in outline, with slightly striated surface, inner edge uniformly and deeply excavated, peaks between excavations tapering to form radial ridges extending about half way across micropylar disc, dividing disc into sectors (Fig. 6H). Number of sectors (or ridges) 6–8 (mean 7.25 $\pm$0.96, n = 4). Area of micropylar disc 20.42–21.40 um (mean 20.98 $\pm$ 0.50 um, n = 3), usually with striated surface.

Morphological comparisons. Table 1 shows the comparisons of 27 attributes of eggs among eight *Anopheles* species, (see Appendix for abbreviations and their equivalents). Four species (i.e. *An. koreicus*, *An. lesteri*, *An. lindesayi japonicus*, *An. sineroides*) have significantly higher float/egg length ratios, from 0.72–0.76, compared with the remaining four species (from 0.51–0.57) ($P \leq 0.05$). *Anopheles koreicus* has a very distinct thin ornamented layer, covering the entire deck. This layer has elongate openings on both ends that extend towards the middle of the deck. It has also a usual round opening at middle part of the deck. *Anopheles belenrae* eggs are significantly longer than those of *An. kleini*, *An. koreicus*, *An. lesteri*, and *An. sinensis* ($P \leq 0.05$). *Anopheles sinensis* eggs have significantly wider middle decks than those of *An. belenrae*, *An. kleini*, *An. koreicus*, and *An. sineroides* ($P \leq 0.05$).

Principal Components. Principal components are useful as a means of identifying the combinations of attributes that provide the important contrasts and differences among usable characters of various species. Six attributes derived from SEM micrographs of the eggs were used: EGGL, EGGW, FLWR, FLELP, FLOA, FLOL (see the Appendix). They were selected using cross tabulations, correlations, parametric statistics and principal component analysis (PROC ANOVA, PROC PRINCOMP; SAS Institute 2003). Of the six derived from the standardized (zero, mean, unit variance), variables, the first three accounted for 89.28% of the variation, and the first two for 70.34% (Table 2). Component 1 carried a heavy positive weighting (Fig. 7) for maximum length of float (FLOL). The attribute egg width (EGGW) had a negative eigenvector in component 1 (Table 2). Component 2 accounted for about 50% less than component 1 (Table 2). Attribute egg width (EGGW) contributed strongly to weightings on this axis (Fig. 8). Component 3 accounted for 18.94% of total variance. The heaviest weightings in this component were mostly egg width (EGGW) and float area (FLOA), although length/width ratio of the float (FLWR) yielded a heavy negative weighting in component 3 (Table 2).

TABLE 2. Partial tabulation of principal components analysis of six attributes of *Anopheles* eggs collected from various sites in the Republic of Korea.*

Principal component	Eigenvalue	% of variance explained	Attribute*					
			EGGL	EGGW	FLWR	FLELP	FLOA	FLOL
1	2.7397	45.66	0.1516	-0.1428	0.0509	0.5577	0.5495	0.5839
2	1.4807	24.68	0.6762	0.5702	0.3795	-0.1899	-0.0716	0.1795
3	1.1364	18.94	0.1733	0.3661	-0.8214	-0.1449	0.3638	-0.0878

*Attribute defined in the Appendix.

Discriminant Functions. When discriminant analysis was applied to six variables to facilitate the separation of the species, the first four functions proved to be significant and the first 2 have 82.75% of the differences among species (Table 3). Four species (*An. belenrae, An. kleini, An. pullus and An. sinensis*) were centered on the negative side of the first discriminant function, whereas the four other species were positive (Figs. 9 and 10). For the second discriminant function, an equal number of species appeared on either side of the centerline, but *An. koreicus* was far removed from the others. Examining the centroids, species differences are found primarily between a group (composed of *An. sineroides, An. lesteri* and *An. lindesayi japonicus*), *An. koreicus*, and another group (composed of *An. kleini, An. sinensis, An. pullus* and *An. belenrae*) (Fig. 10).

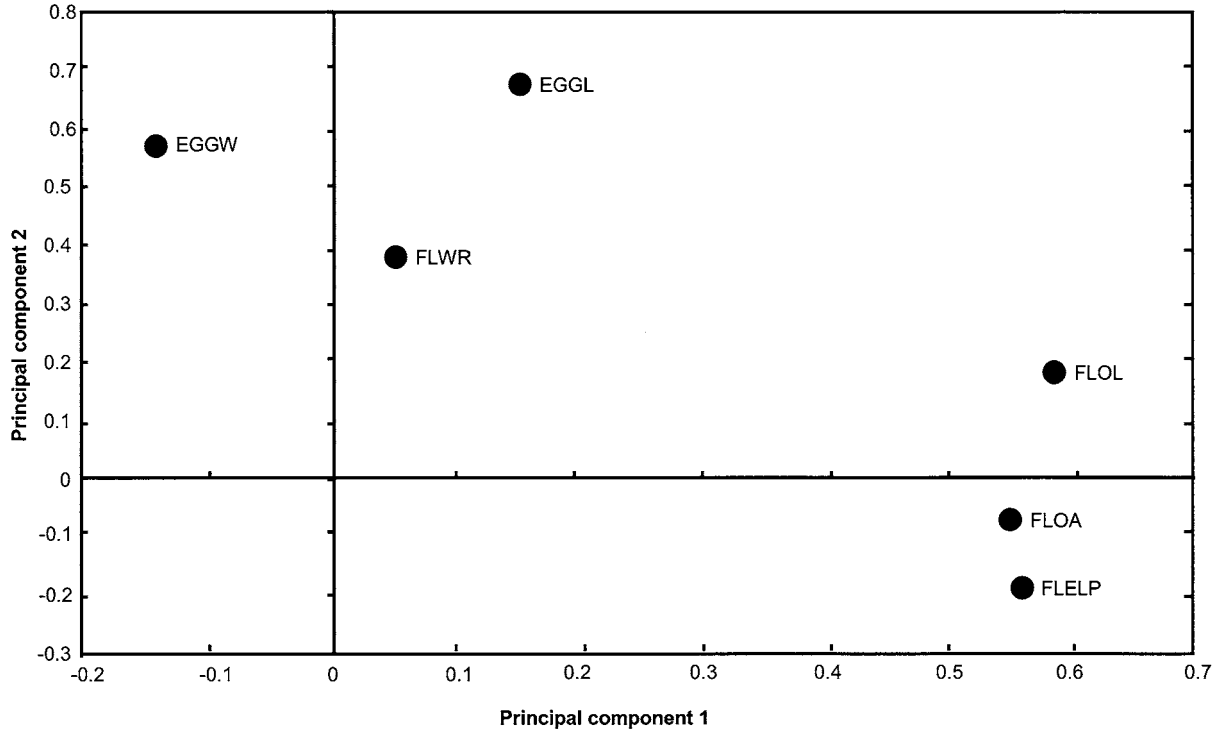

FIGURE 7. Plot of the eigenvectors of the first 2 principal components based on 6 attributes of the eggs of 8 *Anopheles (Anopheles)* species (*An. kleini, An. sinensis, An. sineroides, An. pullus, An. lindesayi japonicus, An. koreicus, An. lesteri, and An.belenrae*). Attributes are defined in the Appendix.

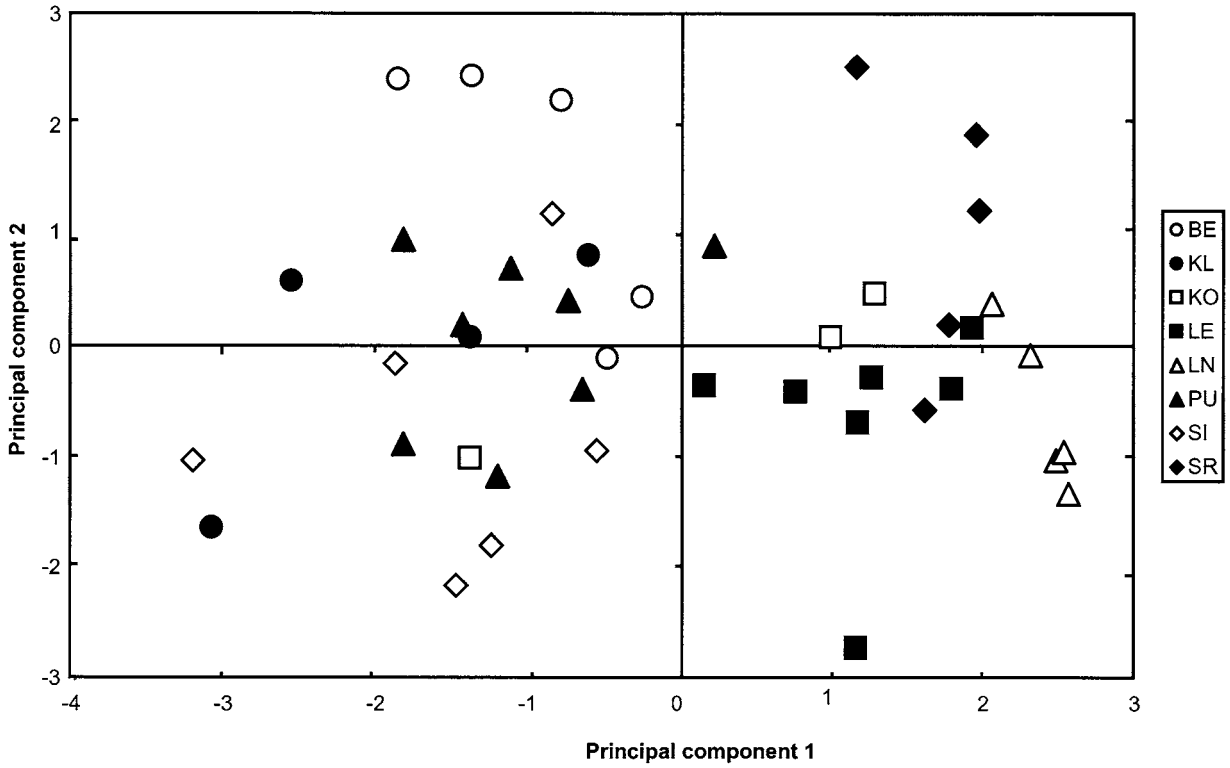

FIGURE 8. Plot of the individual components of the eggs of 8 *Anopheles (Anopheles)* species (BE, *An.belenrae;* KL, *An. kleini;* KO, *An. koreicus;* LE, *An. lesteri*; LN, *An. lindesayi japonicus;* PU, *An. pullus;* SI, *An. sinensis;* and SR, *An. sineroides*).

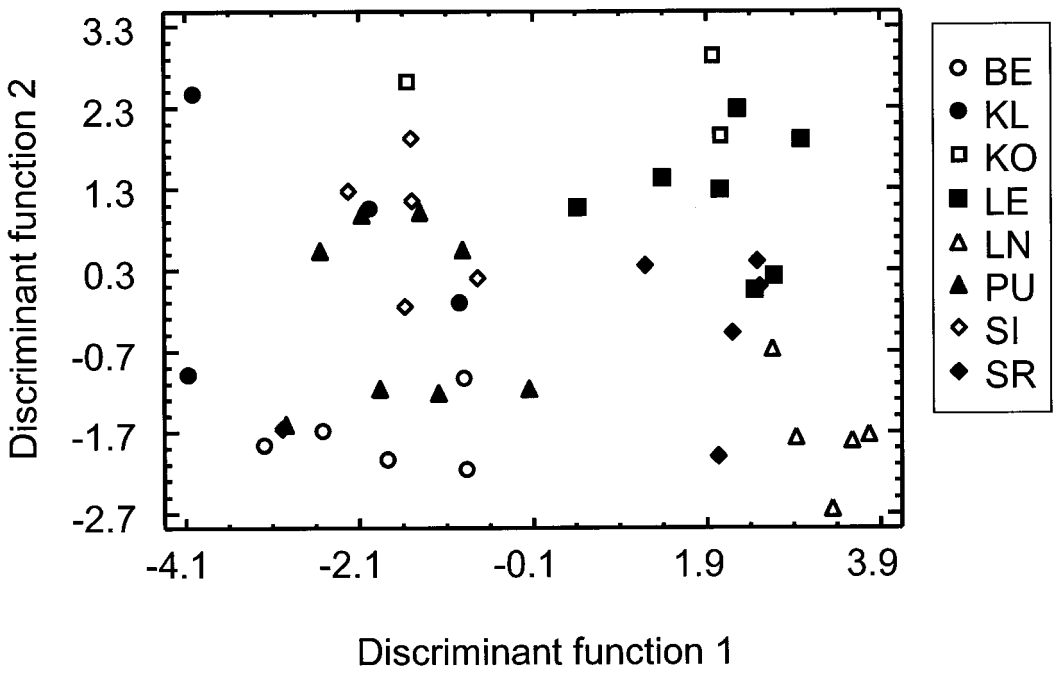

FIGURE 9. Plot of the first 2 individual discriminant functions, based on 6 attributes of the eggs of 8 *Anopheles (Anopheles)* species (BE, *An.belenrae;* KL, *An. kleini;* KO, *An. koreicus;* LE, *An. lesteri*; LN, *An. lindesayi japonicus;* PU, *An. pullus;* SI, *An. sinensis;* and SR, *An. sineroides*).

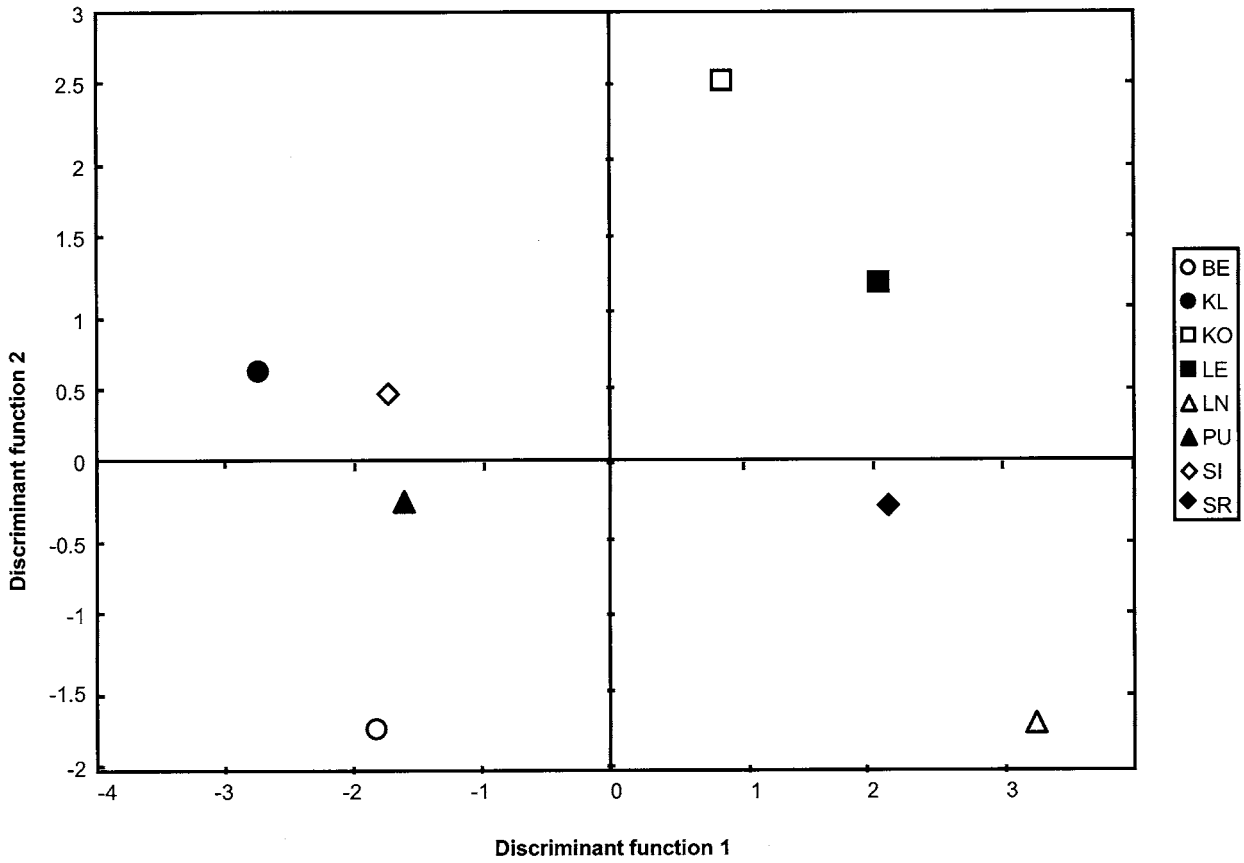

FIGURE 10. Group centroids, by *Anopheles (Anopheles)* species, of the data plotted in Figure 9 (BE, *An.belenrae;* KL, *An. kleini;* KO, *An. koreicus;* LE, *An. lesteri*; LN, *An. lindesayi japonicus;* PU, *An. pullus;* SI, *An. sinensis;* and SR, *An. sineroides*).

Although eggs of *Anopheles* in South Korea are relatively simple in structure, they differ clearly in some attributes at the stereomicroscopic level (Table 1), particularly *An. koreicus*. Multivariate analysis of egg characters indicated that *An. kleini, An. sinensis, An. pullus* and *An. belenrae* generally cluster together (Figs. 8 and 10), and that their separation from *An. lesteri, An. sinensis*, and *An. lindesayi japonicus* on the second principal component was primarily attributable to differences in egg width. Several studies (e.g. Linley 1992, Linley *et al.* 1993, Linley *et al.*1996) used principal component analyses to separate eggs of different *Anopheles* species from different locations. Further studies need to be done to compare morphological differences of mosquito eggs from separate geographical populations of *Anopheles* vectors of malaria, not only from the ROK, but also from other countries. These data maybe useful in revising the taxonomy of various *Anopheles* groups.

TABLE 3. Partial tabulation (non-significant functions omitted) of discriminant analysis of six attributes of *Anopheles* eggs collected from various sites in the Republic of Korea.*

Discriminant function	Eigenvalue	Relative percentage	Chi-squared	df	P
1	5.4907	67.93	147.37	42	0.0000
2	1.7890	14.82	81.90	30	0.0000
3	0.7901	6.72	46.01	20	0.0008
4	0.5227	6.09	25.63	12	0.0121

*Attribute defined in the Appendix.

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Appendix. Definitions of abbreviations (acronyms) of measured or calculated attributes of *Anopheles* eggs.

ATLN=number of lobes of antero-ventral tubercles
ATUN=number of antero-ventral tubercles
DECA=area of deck
DECL=maximum length of deck
DECW=maximum width of anterior deck
DEMW=maximum width of middle deck
DPDN=number of sides of dorsal plastron
DPLA=area of dorsal plastron
DPNLR=area/number of sides ratio of dorsal plastron
EGGL=length of egg
EGGW=width of egg
ELWR=length/width ratio of egg
FLELP=float length as % of egg length
FLEWR=float length/egg width ratio
FLOA=area of float
FLOL=maximum length of float
FLOW=maximum width of float
FLRR=float length/number of ribs
FLWR=length/width ratio of float
FRIN=number of float ribs
FWEWR=float width/egg width ratio
FWRR=float width/number of ribs ratio
MICA=area of micropylar disc
MICDR=area/sector number ratio of micropylar disc
MIDN=number of sectors of micropylar disc
PTLN=number of lobes of postero-ventral tubercles
PTUN=number of postero-ventral tubercles